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Singlet and Triplet State Transitions of Carotenoids in the Antenna Complexes of Higher-Plant Photosystem I[†]

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ABSTRACT: In this work, the spectroscopic characteristics of carotenoids associated with the antenna complexes of Photosystem I have been studied. Pigment composition, absorption spectra, and laser-induced triplet-minus-singlet (T–S) spectra were determined for native LHCI from the wild type (WT) and *lut2* mutant from *Arabidopsis thaliana* as well as for reconstituted individual Lhca WT and mutated complexes. All WT complexes bind lutein and violaxanthin, while β -carotene was found to be associated only with the native LHCI preparation and recombinant Lhca3. In the native complexes, the main lutein absorption bands are located at 492 and 510 nm. It is shown that violaxanthin is able to occupy all lutein binding sites, but its absorption is blue-shifted to 487 and 501 nm. The “red” lutein absorbing at 510 nm was found to be associated with Lhca3 and Lhca4 which also show a second carotenoid, peaking around 490 nm. Both these xanthophylls are involved in triplet quenching and show two T–S maxima: one at 507 nm (corresponding to the 490 nm singlet absorption) and the second at 525 nm (with absorption at 510 nm). The “blue”-absorbing xanthophyll is located in site L1 and can receive triplets from chlorophylls (Chl) 1012, 1011, and possibly 1013. The red-shifted spectral component is assigned to a lutein molecule located in the L2 site. A 510 nm lutein was also observed in the trimers of LHCII but was absent in the monomers. In the case of Lhca, the 510 nm band is present in both the monomeric and dimeric complexes. We suggest that the large red shift observed for this xanthophyll is due to interaction with the neighbor Chl 1015. In the native T–S spectrum, the contribution of carotenoids associated with Lhca2 is visible while the one of Lhca1 is not. This suggests that in the Lhca2–Lhca3 heterodimeric complex energy equilibration is not complete at least on a fast time scale.

Carotenoids play several roles in photosynthesis: they act as accessory pigments in collecting light and transfer excitation energy to the Chls;¹ they protect the system by dissipating the Chl triplets and working as oxygen scavengers (1); they are essential for the stability of the Lhc proteins (2); and they are involved in the energy dissipation from the chlorophyll singlet excited states (3). Although ~600 carotenoids have been described in nature, only a small

number are found in higher-plant antenna complexes, while their composition is highly conserved, suggesting a specific role for each of them. The recently published structure of LHCII (4), the major antenna complex of PSII, shows the location of four carotenoid binding sites. Two of them (L1 and L2) are located in the center of the macromolecule where they host lutein in the protein purified from leaves. Nevertheless, in carotenoid biosynthesis mutants or in recombinant protein reconstituted in the absence of lutein, sites L1 and L2 can also bind violaxanthin and/or zeaxanthin (5–7). The xanthophylls bound to these sites act in both energy transfer and Chl triplet quenching (8). Lutein in site L1 is responsible for an absorption band at ~494 nm and a triplet-minus-singlet (T–S) maximum around 506–507 nm (9–13). The spectroscopic characteristics of the lutein in site L2 strongly depend on the aggregation state of the complex. In monomeric LHCII, lutein in L2 exhibits singlet absorption at around 490 nm (14) and triplet absorption at 506 nm (8), while in trimeric complexes, the S_{2,0}–S_{0,0} absorption is red-shifted to 510 nm and the T–S maximum to 525 nm (9, 10). The origin of these changes is unknown, but several possibilities have been proposed (15). The xanthophyll bound

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¹ Abbreviations: β -DM, *n*-dodecyl β -D-maltoside; Car, carotenoid; Chl, chlorophyll; Lhc, light-harvesting complex; LHCI and -II, light-harvesting complex of Photosystem I and II, respectively; PS, Photosystem; T–S, triplet-minus-singlet.

Table 1: Pigment Composition^a

	Chl <i>a</i> /Chl <i>b</i>	Chl/Cars	violaxanthin	lutein	β -carotene	zeaxanthin	Chl <i>a</i> + Chl <i>b</i>
LHCI WT	3.4	4.4	2.3	4.9	1.8		40
LHCI- <i>lut2</i>	3.6	4.4	6.7	—	1.8	0.6	40
Lhca1	4.0	3.3	1.0	1.8			10
Lhca2	1.85	5.0	0.5	1.5			10
Lhca3	6.0	3.5	0.7	1.6	0.5		10
Lhca4 WT	2.4	4.8	0.4	1.7			10
Lhca4 N47H	2.4	4.9	0.3	1.7			10
Lhca4 N156F	1.9	4.6	0.3	1.6			9

^a The pigment composition of the samples analyzed in this work. The data are normalized to the number of Chls reported in the last column. The maximal standard deviation is 0.1. The first two samples are native, purified from plants; the others are reconstituted complexes.

to site L1 has been shown to be essential for structure stabilization: its occupation by either violaxanthin, zeaxanthin, or lutein is needed for the folding of the monomeric complex (16), while the latter xanthophyll is the only one which allows trimerization (17, 18). A third carotenoid binding site (N1), occupied by neoxanthin, is located near helix C of LHCII (19). This carotenoid has been shown to be active in transferring singlet energy to Chl, but it does not seem to accept Chl triplets, possibly because nearest-neighbor chromophores are Chls *b*, having a low probability of forming triplets (14, 20) since energy transfer from Chl *b* to Chl *a* is much faster (10^3 -fold) than triplet formation. The occupancy of this site is dispensable for the stability of the complex (5). The fourth site (V1) is located at the periphery of the complex and under normal conditions accommodates violaxanthin and lutein (21, 22). The carotenoids in this site are not involved in energy transfer or triplet quenching (22). Bassi and Caffarri (23) suggested that the V1 site is a source of violaxanthin ready to be converted in zeaxanthin under stress conditions. Whereas the carotenoid organization in LHCII and in the minor antenna complexes of PSII has been extensively studied, little is known about the carotenoids in Lhca complexes. The antenna complex of PSI is composed by four homologous Lhca proteins (Lhca1–4), located on one side of the PSI core complex (24). The structure at 4.4 Å reveals the location of Chls within the pigment–protein complexes but does not resolve carotenoid molecules. Nevertheless, the high degree of sequence homology with LHCII (genes *Lhcb1*–3) suggests a similar organization. In this study, we used a native LHCI preparation from wild-type (WT) and mutant plants and recombinant Lhca complexes to determine the characteristics of singlet and triplet absorptions of the Cars associated with these complexes.

EXPERIMENTAL PROCEDURES

Purification of LHCI from WT and *lut2* Plants. LHCI complexes were isolated by solubilizing PSI–LHCI complexes (at a concentration of 0.3 mg/mL) from *Arabidopsis thaliana* (WT and *lut2* mutant) by a treatment using 1% β -DM and 0.5% Zwittergent-16 and purified by sucrose density gradient centrifugation using the method of Croce et al. (25).

Mutagenesis and in Vitro Reconstitution. cDNA of Lhca4 from *A. thaliana* (26) was mutated with the QuickChange site-directed mutagenesis kit, by Stratagene. WT and mutants apoproteins were isolated from the SG13009 strain of *Escherichia coli* transformed with constructs following a protocol previously described (27). Reconstitution and purification of protein–pigment complexes were performed as described in ref 26.

Protein and Pigment Concentration. HPLC analysis was performed as described in ref 28. The chlorophyll/carotenoid ratio and the Chl *a*/Chl *b* ratio were quantified independently by fitting the spectrum of acetone extracts with the spectra of individual purified pigments (29).

Spectroscopy. The absorption spectra at room temperature and 77 K were recorded using an SLM-Aminco DK2000 spectrophotometer, and samples were resuspended in 10 mM Hepes (pH 7.5) (60% glycerol for the 77 K measurements) and 0.06% β -DM. The scan rate was 100 nm/min, the sampling step 0.4 nm, and the optical path length 1 cm.

Light-induced absorbance changes were recorded with a home-built high-sensitivity laser-based spectrophotometer as described in ref 30. The light pulse with a time duration of 5 ns of a Surelite Q-switched Nd:YAG laser (Continuum) is used to pump a Surelite OPO (BBO I crystal, Continuum) which produces coherent, broadband tunable radiation from 420 to 590 nm. These light pulses are used to detect light-induced absorbance changes in the time range from 10 ns to 10 μ s with a high signal/noise ratio (10^{-5} OD). Excitation light is provided by dye laser emission (DCM 650) in a home-built dye cell which is pumped by a second harmonic Minilite Nd:YAG laser (Continuum). The excitation pulses have an intensity of ~ 1.5 mJ, a wavelength of 640 ± 10 nm, and a duration of 5 ns. Three Schott BG39 (3 mm) optical filters and a low pass dielectric filter (transmission of $<10^{-3}$ at 600 nm) are placed in front of each detector. The measurements were performed at room temperature.

Data Analysis. For a given wavelength, the kinetics of the absorbance change was recorded with variable delay times from 5 ns to 50 μ s between the actinic and the detection light pulses. For the global fit analysis, the data were analyzed in the time interval between 100 ns and 10 μ s, taking into account only the carotenoid triplet decay signal. GraphPad PRISM (GraphPad Software) was used to globally analyze the kinetics obtained between 420 and 580 nm. All the T–S absorbance changes were normalized to the same OD at 640 nm.

RESULTS

Pigment Content. LHCI was purified from *A. thaliana* WT plants and the *lut2* mutant which does not contain lutein (31). The pigment composition is reported in Table 1. In the WT complex, three carotenoids are present: lutein, the most abundant, violaxanthin, and β -carotene. In LHCI-*lut2*, lutein is not present, and this absence is compensated by an increased amount of violaxanthin and, to a lesser extent, zeaxanthin. The Chl *a*/Chl *b* ratio and the Chl/Car ratio are very similar in the two preparations, indicating that the total

number of carotenoid molecules bound to the complexes is unchanged. This implies that binding sites are not completely selective, thus allowing lutein and violaxanthin (and zeaxanthin) to occupy the same sites. The amount of β -carotene is identical in the two samples, suggesting a more specific binding site for this pigment.

The native preparation of LHCI contains all four Lhca complexes, and thus, it is not possible to determine the characteristics of the carotenoids bound to individual gene products. Toward this end, recombinant Lhca complexes were produced by overexpression of the apoproteins in bacteria and reconstitution in vitro with thylakoid pigment extracts. These complexes exhibited a monomeric aggregation state. The pigment composition for the pigment–protein complexes used in this work is reported in Table 1.

In agreement with the data for native LHCI, lutein is the most abundant xanthophyll in all Lhc complexes. Violaxanthin is also present in all of them, while β -carotene was found only in the Lhca3 complex. Neoxanthin which is a component of the antenna complexes of PSII, although present in the pigment mix used for the reconstitution, does not bind to Lhca proteins, in agreement with the composition of the native complexes and of purified stroma membranes (32, 33). The set of samples analyzed in this work is completed by two recombinant proteins carrying mutations affecting Chl binding sites located in close connection with L2 and L1 carotenoid binding sites. In Lhca4 N47H, the Chl 1015 (nomenclature from ref 24) binding site is modified by the substitution of N with H, which does not change the pigment composition but abolishes the red forms (34). Lhca4 N156F lacks Chl *a* 1012 as shown by the decrease in the Chl *a*/Chl *b* ratio, while the carotenoid composition is not affected by the mutation.

Absorption Spectra. To investigate the spectral properties of the carotenoids in the Lhca complexes, the absorption spectra at 77 K were recorded. In Figure 1, the blue region of the spectra of LHCI WT and LHCI-*lut2* complexes are reported together with their second derivatives in the carotenoid absorption region. In the WT complex, minima in the second-derivative spectrum are observed at 492, 509, and 517 nm. The second derivative of LHCI-*lut2* shows a very different pattern, with minima at 484, 487, 501, and 517 nm, suggesting that the bands at 492 and 509 nm present in the WT sample are associated with lutein. In the same binding sites, the violaxanthin seems to absorb at 487 and 501 nm. This is in agreement with the spectra of the same carotenoids in organic solvents, where the violaxanthin is blue-shifted at least 5 nm as compared to the lutein. The conservation of the signal at 517 nm in the two samples suggests that it is due to β -carotene.

For a more detailed analysis of the absorption characteristics of the individual carotenoids, the absorption spectra of the reconstituted WT complexes and of the two mutants of Lhca4 affected in Chls located in the proximity of the L1 and L2 site were also recorded at 77 K and are presented in Figure 2 together with their second derivative. The contributions of the $S_{2,0}$ – $S_{0,0}$ transition of the carotenoids can be detected at 498 and 488 nm in Lhca1, 493 nm in Lhca2, 491 and \sim 510 nm in Lhca3, and 490 and 510 nm in all three Lhca4 samples.

To determine the contribution of each chromophore, the absorption spectra of the complexes were described in terms

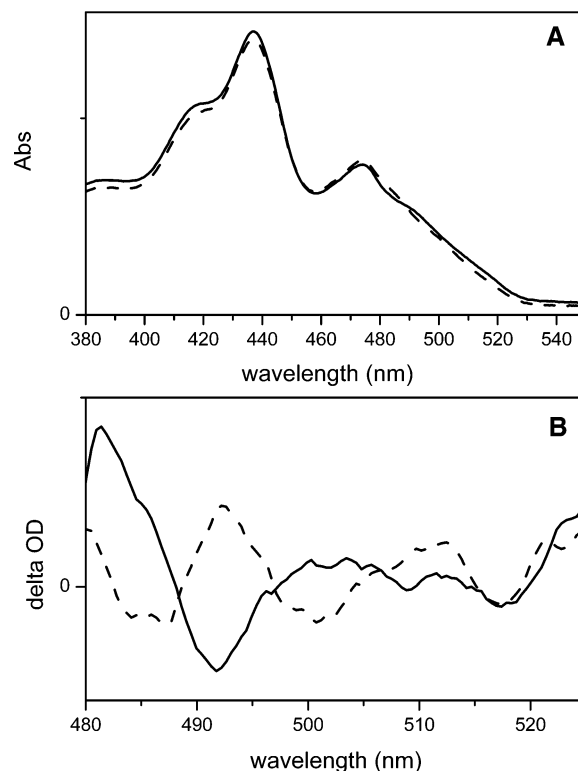


FIGURE 1: Absorption characteristics of LHCI WT (—) and LHCI-*lut2* (---) complexes at 77 K. (A) Blue region of the absorption spectra. The spectra are normalized to the Chl content in the Q_y region. (B) Second derivatives of the carotenoid absorbing region (480–530 nm) of the spectra in panel A.

of absorption of individual pigments, using the absorption spectra of pigments in a protein environment (35). The absorption spectra of several mutants of Lhca complexes lacking Chls but also affected in the carotenoid binding (36, 37) were analyzed to determine the absorption maxima of the individual carotenoids (data not shown). These results were used as starting parameters in the fitting. The fitting program allows us to shift the spectra on the wavelength scale and to vary their intensity. The best fitting also reproduces the stoichiometry of the pigments. The results are reported in Figure 3, and Table 2 summarizes the absorption maxima of the carotenoids in the different Lhc complexes. Two distinct lutein absorptions can be distinguished at \sim 489 and 500–510 nm. The absorption of violaxanthin was found between 492 and 496 nm in all complexes.

T–S Spectra. The main role of carotenoids within the photosynthetic pigment–protein complexes is photoprotection against singlet oxygen. This is achieved in a dual way: singlet oxygen scavenging and prevention of singlet oxygen formation by acceptance of triplet states from Chl *a* and dissipation of excess energy into heat. It has been suggested that the red forms in Lhca4 play a role in enhancing the triplet protection (38).

Triplet formation in Lhca native and recombinant complexes was studied by flash-induced transient absorption under aerobic conditions: 5 ns flashes excited Chl *a* and *b* at 640 ± 10 nm, and absorption changes were detected in the wavelength range of 420–600 nm. The kinetics in the 420–580 nm range, of LHCI samples from WT and *lut2* plants under aerobic conditions, were fitted with a single

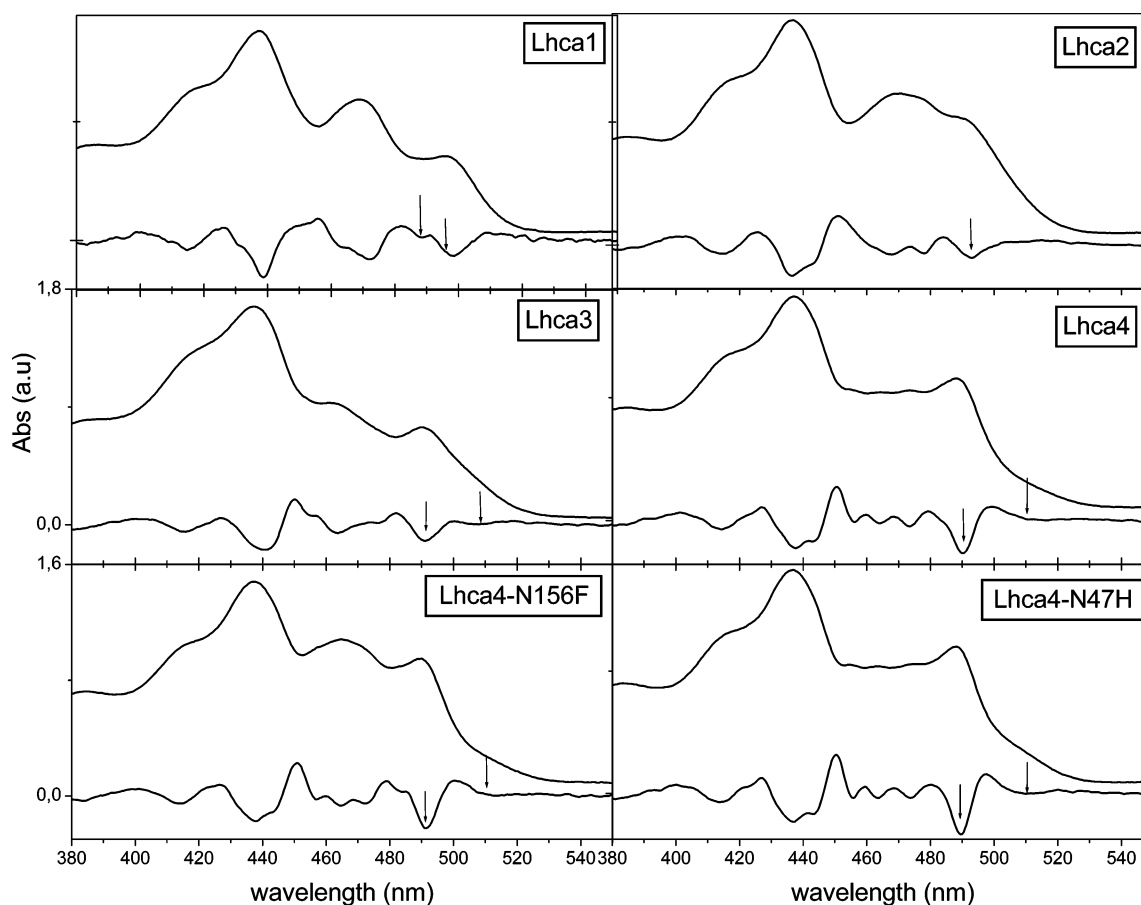


FIGURE 2: Absorption spectra in the carotenoid absorption region at 77 K of recombinant Lhca complexes and second derivatives. The arrows indicate the minima related to the carotenoid absorption.

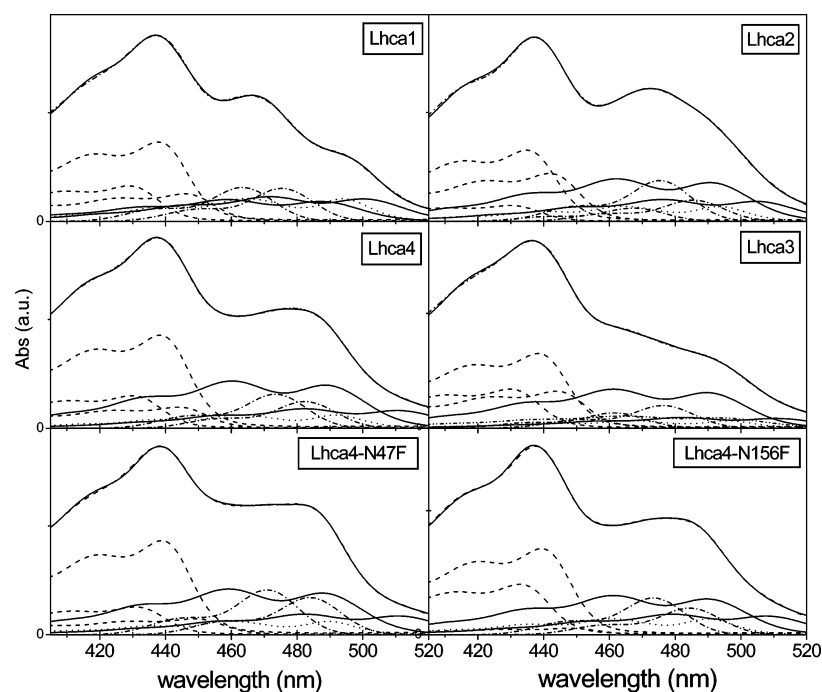


FIGURE 3: Fitting (---) of the blue region of the absorption spectra at room temperature (—) of reconstituted WT and mutants of Lhca proteins with the spectra of individual pigments: Chl *a* (---), Chl *b* (---), lutein (— · —), violaxanthin (···), and β -carotene (---).

component having a lifetime of 2.38 μ s in both samples (Figure 4). The LHCI WT T-S spectrum shows two maxima peaking at 510 and 530 nm. The LHCI-*lut2* T-S spectrum shows a maximum at 513 nm and a small shoulder at approximately 530 nm. The difference spectrum between WT

and *lut2* T-S spectra, after normalization to the same excitation intensity, is presented in Figure 4. The major components are at 515 (—) and 532 nm (+), suggesting that the latter peak originates from lutein, while the major T-S contribution of violaxanthin is detected at 515 nm.

Table 2: Absorption Maxima (nanometers) of Carotenoids Associated with the Recombinant Lhca Proteins^a

	violaxanthin	lutein	β -carotene
Lhca1	493.8	487, 500	
Lhca2	493.3	490, 504	
Lhca3	495.8	489.5, 510.5	492.5
Lhca4	491.8	488.5, 510.5	

^a The values were obtained by fitting the absorption spectrum with the spectra of pigments in proteins.

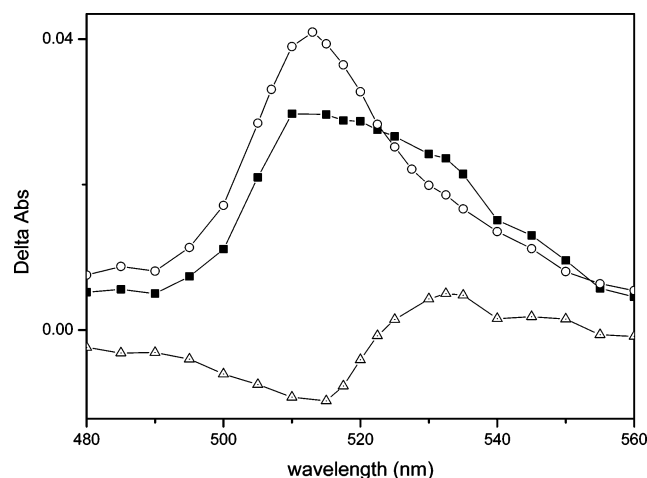


FIGURE 4: T–S spectra of LHCI-WT (■) and LHCI-*lut2* (○) samples at room temperature under aerobic conditions. Spectra of the 2.38 μ s components were obtained by global analysis. The difference spectrum is also reported (Δ). The spectra are normalized in such a way that the amount of excitation in the two samples is the same.

In Figure 5, the T–S spectra of Lhca1–4 monomeric complexes under aerobic conditions at room temperature are reported. The decay of the carotenoid triplet state is very similar and monoexponential for all the complexes with values between 2.25 and 2.67 μ s (see Table 3). In contrast, the shapes of the spectra differ considerably. Lhca1 exhibits a maximum at 510 nm, and its spectrum is very similar to the T–S spectrum of monomeric LHCII (8, 13). In the case of Lhca2, the spectrum is broader and exhibits a peak at 513 nm and a shoulder at approximately 525 nm. This latter component is more pronounced for the Lhca3 complex, and it becomes dominant in Lhca4, its spectrum exhibiting a 525 nm peak and a clear shoulder at 507 nm.

When the measurements were performed under anaerobic conditions (data not shown), the decay times of the T–S signals were longer, approximately 9 μ s, an observation which has also been reported for LHCII (9). The spectra under aerobic conditions are identical to those under anaerobic conditions, implying that there is no preference for direct quenching of triplet states by oxygen.

The presence of two spectral components for Lhca2, Lhca3, and Lhca4 indicates the contribution of two carotenoid molecules, with different absorption characteristics, to the T–S spectrum. To assign each of these to individual xanthophylls in specific binding sites, T–S spectra were measured on two mutated Lhca4 complexes. The Lhca4 N47H mutant is affected in Chl 1015, localized close to the L2 carotenoid binding site; Lhca4 N156F is affected in Chl 1012, close to the L1 carotenoid binding site. The T–S

spectra of the two mutants obtained by global analysis are given in Figure 6.

The amplitude of the two spectral components peaking at 507 and 527.5 nm changes in the two mutants as compared to that in WT: Lhca4 N47H shows a maximum at 507 nm and a reduced intensity at 525 nm, while the spectrum of Lhca4 N156F shows a clear maximum at 527.5 nm. These results indicate that the carotenoid in the L2 site is responsible for the 525 nm T–S signal, while the 507 nm T–S maximum is due to the xanthophyll in the L1 site. This is in agreement with optically detected magnetic resonance (ODMR) data which showed that the 525 nm T–S signal arises from a carotenoid connected to the red forms (38). Interestingly, under aerobic conditions, the lifetime corresponding to the carotenoid T–S decay of the Lhca4 N47H mutant is identical to that of the WT, while it is shorter for the Lhca4 N156F mutant. This is not the case under anaerobic conditions where Lhca4 N156F exhibits the same decay lifetime as WT (data not shown). The difference in decay times under aerobic and anaerobic conditions has also been observed in the case of the LHCII complex, and it has been attributed to an enhancement of Car triplet intersystem crossing by oxygen (39). The shorter lifetime observed for the Lhca N156F mutant as compared to that of LHCI WT and the Lhca4 N47H mutant can be due to differential accessibility for oxygen (40). This is understandable taking into account that this mutant misses Chl 1012. Inspection of the LHCII structure (4) shows that carotenoids in sites L1 and L2 are deeply buried in the pigment–protein structure. The lack of Chl 1012, however, may leave an open channel via which oxygen can reach the carotenoids.

DISCUSSION

The analysis of LHCI purified from WT plants shows that only three carotenoid species are coordinated to the complexes under normal conditions, lutein, β -carotene, and violaxanthin. The native LHCI preparation contains the four Lhca complexes in dimeric form, each dimer binding approx 20 Chl molecules and 4.4 carotenoid molecules (26). Thus, the whole LHCI complex binds 40 Chls, 2.3 violaxanthins, 4.9 luteins, and 1.8 β -carotenes. Each monomer binds 2.2 Cars per polypeptide on average. Similar results were obtained from the analysis of the LHCI-*lut2* preparation. On the basis of 40 Chls, 6.7 violaxanthins, 1.8 β -carotenes, and 0.6 zeaxanthin molecule were found, implying that all the five lutein binding sites of LHCI WT can also be occupied by violaxanthin. The recombinant proteins bind the same carotenoid species as the native complexes. The fact that neoxanthin was absent in recombinant pigment–protein complexes, although present in the reconstitution mixture during refolding, indicates that the neoxanthin binding site as found in LHCII (4, 19) is empty or nonexistent in Lhca complexes. As shown previously, Lhca2 and Lhca4 each coordinate two xanthophyll molecules, while Lhca1 and Lhca3 each coordinate three (26, 41). In total, the recombinant proteins bound 10 carotenoid molecules, one more than the number found for the native preparation: 2.7 violaxanthins, 6.5 luteins, and 0.6 β -carotene. The amount of β -carotene is clearly lower than in the native complexes. One explanation is that the binding of β -carotene is stabilized by protein–protein interaction. Indeed, the Lhca1–Lhca4 dimer was found to coordinate β -carotene, while monomeric Lhca1 and

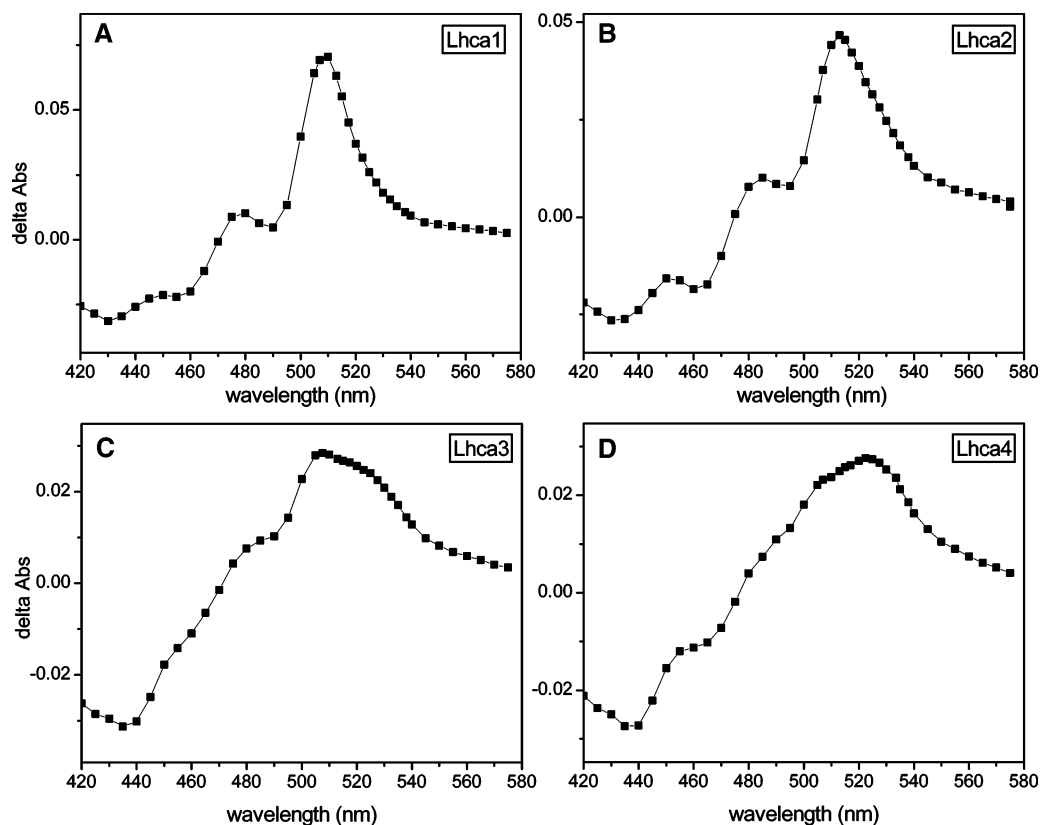


FIGURE 5: T–S spectra of Lhca WT reconstituted complexes at room temperature under aerobic conditions: (A) spectrum of the 2.25 μ s component of Lhca1, (B) spectrum of the 2.55 μ s component of Lhca2, (C) spectrum of the 2.41 μ s component of Lhca3, and (D) spectrum of the 2.67 μ s component of Lhca4. The spectra were obtained by global analysis. The spectra are normalized in such a way that the amount of excitation is the same.

Table 3: Decay Lifetimes^a

sample	τ (μ s)	sample	τ (μ s)
Lhca1	2.25 ± 0.06	Lhca4 N47H	2.61 ± 0.09
Lhca2	2.55 ± 0.02	Lhca4 N156F	1.95 ± 0.02
Lhca3	2.41 ± 0.07	LHCI WT	2.38 ± 0.33
Lhca4	2.67 ± 0.06	LHCI- <i>lut2</i>	2.38 ± 0.11

^a Decay lifetimes of the T–S spectra were obtained by global analysis of a data set consisting of time traces measured at different wavelengths.

Lhca4 were not (26). The fact that the amount of β -carotene is the same in WT and *lut2* plants and that it corresponds to approximately 2 molecules suggests that two of the carotenoid binding sites in Lhca complexes are selective for β -carotene.

Singlet State Transitions. The analysis of the absorption spectra of Lhca complexes shows two major carotenoid absorption bands, both red-shifted with respect to the absorption of xanthophylls in organic solvent (the red-most transition of lutein in acetone is located at 478 nm), one at around 490 nm and the second at 510 nm. The absence of the 510 nm signal in the LHCI-*lut2* sample indicates that this signal is due to lutein. In LHCI-*lut2*, a new signal appears at 500 nm, suggesting that the violaxanthin which substitutes for lutein in the site conferring the 510 nm absorption is also strongly red-shifted (~ 28 nm as compared to the spectrum in acetone). The analysis of the reconstituted complexes reveals that lutein is present in Lhca3 and Lhca4. In Lhca2, the shift seems to be less pronounced and a form at ~ 504 nm can be detected. In the case of Lhca1, the red-most lutein peaks at 500 nm. A second carotenoid

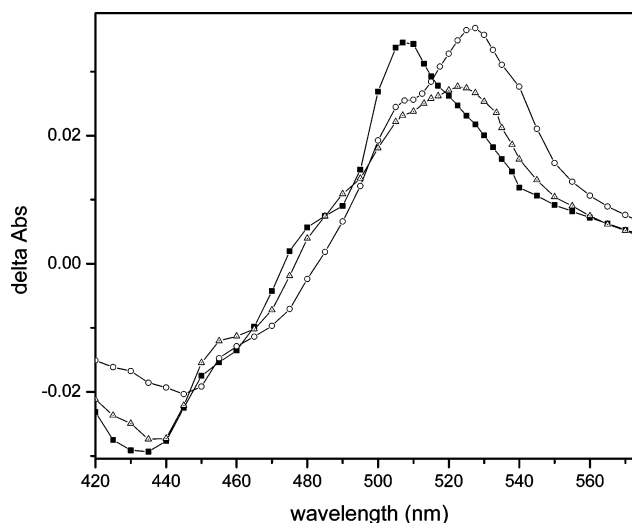


FIGURE 6: T–S spectra at room temperature under aerobic conditions of the 2.67 μ s component of Lhca4 WT (Δ), the 2.61 μ s component of Lhca4 N47H (\blacksquare), and the 1.94 μ s component of Lhca4 N156F (\circ). The spectra were obtained by global analysis. The spectra are normalized in such a way that the amount of excitation is the same.

contribution can be detected at ~ 490 – 494 nm in all complexes; this absorption is associated with both lutein and violaxanthin.

Triplet State Transitions. The T–S spectrum of Lhca1 is virtually identical to that previously described for monomeric LHCII (8), with a maximum at 510 nm and minima at 490 and 460 nm. The presence of these two components suggests that the 510 nm T–S signal originates from a carotenoid

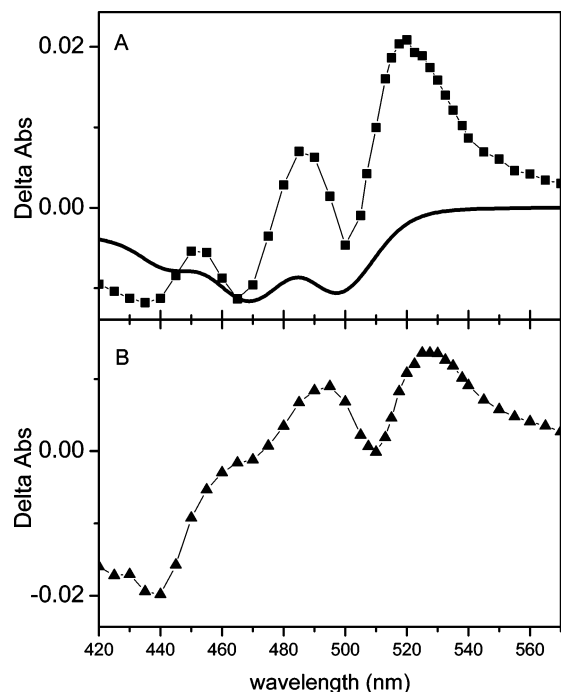


FIGURE 7: (A) T-S Lhca2-minus-Lhca1 difference spectrum (■). The spectrum of lutein multiplied by -1 and shifted to 500 nm is also reported (—). The relative amplitude of the T-S spectra of Lhca2 and Lhca1 before subtraction was 1.36. (B) T-S Lhca3-minus-Lhca1 difference spectrum. The spectra were normalized to the maximum before subtraction.

molecule absorbing around 490 nm. The negative signal at 460 nm is likely to represent the second vibronic transition of the carotenoid singlet spectrum, which in solution is blue-shifted 30 nm with respect to the lowest-energy transition. An additional negative signal at 430 nm mainly arises from Chl *a* molecules which experience absorbance changes due to triplet formation on a neighboring carotenoid (9, 39, 42).

The T-S spectrum of Lhca2 is broadened in the red as compared to that of Lhca1 and looks very similar to the spectrum of the trimeric LHCII complex (9), exhibiting two components, one peaking more to the blue at ~ 510 nm and a second one more to the red at ~ 520 nm, while Lhca1 exhibits only the former. Subtracting the Lhca1 spectrum from that of Lhca2, in such a way that only the 520 nm feature remains, leads to the results in Figure 7. The Lhca2-minus-Lhca1 T-S difference spectrum (Figure 7A) exhibits a maximum at 520 nm and minima at 500 and 470 nm, suggesting that in Lhca2 an additional carotenoid spectral form with singlet absorption at ~ 500 nm is responsible for the 520 nm triplet spectrum.

Two components can be detected in the T-S spectrum of Lhca3; the difference spectrum with Lhca1 (Figure 7B) shows a maximum at 527 nm and a minimum at 510 nm, suggesting that the carotenoid responsible for the 527 nm T-S signal has the lowest-energy singlet transition at 510 nm. An additional positive component at 495 nm is visible in the difference spectrum, possibly representing the high vibronic state of the triplet spectrum. Similar results were obtained for Lhca4, in which the 525 nm form dominates the spectrum. A component at 525 nm in Lhca4 was resolved also by ODMR measurements at 1.8 K (38).

To determine the contribution of each complex to the T-S spectrum of the native preparation, the spectra of the Lhca

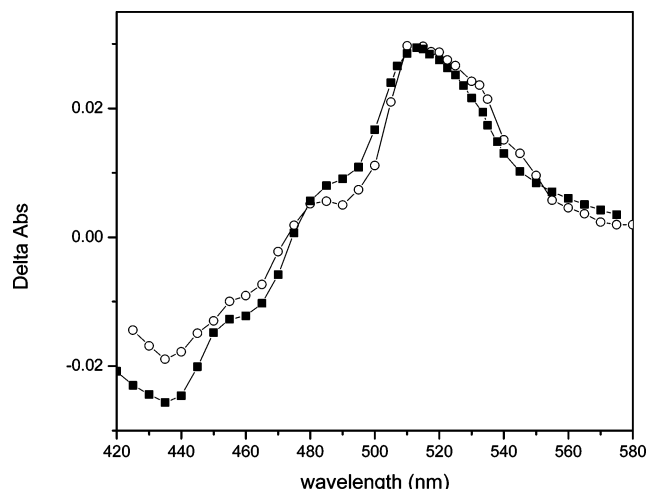


FIGURE 8: Comparison between the T-S spectra of LHCI WT (○) and the sum of the T-S spectra of Lhca2 (multiplied for 0.8), Lhca3, and Lhca4 (■). For the sum, the T-S spectra of individual Lhca complexes were normalized to their relative intensity in the absorption at 640 nm.

complexes were summed, and the best match (in both intensity and shape) between the sum and the native T-S spectrum is obtained when only Lhca2, Lhca3, and Lhca4 are taken into account (Figure 8). Although the ratio in which each complex contributes to the spectrum cannot be determined precisely, it is clear that there is not a contribution of the Lhca1 complex to the spectrum of native LHCI. The carotenoid in Lhca1 does not seem to participate in the triplet protection in the native preparation, although the sample clearly contains Lhca1 (26). This is consistent with the previous finding that Lhca1 transfers most of its energy to Lhca4 with which it forms a functional dimer (43). The carotenoids in Lhca2 are apparently accepting Chl triplets in the native preparation. This would suggest that in the expected Lhca2-Lhca3 dimer part of the excitation energy still resides on the Lhca2 complex, possibly due to the fact that the interaction between these complexes is weaker than that observed for the Lhca1-Lhca4 dimer. This is consistent with the observation that detergent treatment is more effective in monomerizing the Lhca2-Lhca3 dimer than the Lhca1-Lhca4 dimer (26).

The spectrum of native LHCI is slightly red-shifted as compared to the sum of the individual Lhca complexes. A signal at 532 nm is detectable in the T-S spectrum of WT, possibly originating from the carotenoid absorbing at 517 nm. This seems consistent with the finding that the T-S maximum is always approximately 15 nm red-shifted as compared to the singlet spectrum. The 517 nm absorption has been tentatively attributed to β -carotene, being present also in LHCI-*lut2*. The T-S spectrum of LHCI-*lut2* indeed conserves a signal in this region. Moreover, no clear 517 nm absorption band or a 532 nm T-S band is detectable in the recombinant proteins, which contain reduced amounts of β -carotene as compared to the WT. This leads to the conclusion that β -carotene in the native preparation is active in triplet quenching. The carotenoid triplet decay times in all Lhca complexes are very similar to each other and also similar to the decay in LHCII (8, 13), indicating a similar environment.

Location of the Carotenoids. Four carotenoid binding sites have been observed in the structure of LHCII, L1 and L2

located in the center of the protein forming a crossbrace between helices A and B, N1 occupied by neoxanthin and located near the C helix, and V1 occupied by lutein and violaxanthin and located at the periphery of the complex (4, 5, 21, 22). It has been suggested that the neoxanthin binding site is very selective for this xanthophyll, and the absence of neoxanthin in Lhca complexes suggests that this site is empty or nonexistent in these complexes, with the possible exception of Lhca1 in which a small amount of neoxanthin has been observed (44). The three remaining sites present in LHCII can all be occupied by both lutein and violaxanthin, although L1 and L2 clearly have a preference for the former (5, 6). According to the pigment composition, Lhca2 and Lhca4 coordinate only two carotenoids with a violaxanthin/lutein ratio of 1/3. These data support the view that the two carotenoids associated with Lhca2 and Lhca4 are bound to the two innermost sites, L1 and L2. In Lhca3 and Lhca1, three carotenoids are present. Lhca1 exhibits an increased content of both lutein and violaxanthin as compared to Lhca4, suggesting that the additional binding site corresponds to site V1 in LHCII, although the possibility of these xanthophylls being in the N1 site cannot be excluded. In Lhca3, the main difference with respect to Lhca2 and Lhca4 is the presence of β -carotene. On the basis of mutation analysis, it has been proposed that Lhca3 accommodates β -carotene in the L2 site (37). However, β -carotene has never been found in other Lhc proteins. Thus, we cannot rule out the possibility that Lhca3 exhibits a special β -carotene binding site, different from those found in LHCII.

To assign spectral properties to each carotenoid in each binding site, two mutants of Lhca4 were analyzed. Lhca4 N47H is affected in the binding ligand for Chl 1015 which is the nearest neighbor of carotenoid in site L2. Moreover, this complex loses the 732 nm red-shifted emission (34). Lhca4 N156F loses Chl 1012, which is the nearest neighbor to the carotenoid molecule in site L2. These two mutants show the same carotenoid composition as WT, indicating that the mutation did not affect the binding of xanthophyll or the specificity of the binding sites. The analysis of the absorption spectra reveals that the absorption maxima of the carotenoids are practically unchanged in the case of Lhca4 N47F, and only a small blue shift of the 510 nm lutein to 507 nm can be observed in the Lhca4 N47H mutant. On the other hand, clear differences can be observed in the T–S spectra. LHCI N47H exhibits a decrease in the 525–532 nm contribution and an increase in the 507 nm form. This indicates that in the mutant fewer triplets are transferred from Chls to lutein at 525 nm than in WT Lhca4, leading to the conclusion that the carotenoid responsible for the 525 nm T–S spectrum is located in site L2, near Chl 1015 whose absorption has been changed by the mutation. The reduction in the intensity of the 525 nm form is the result of a different distribution of the excitation energy among Chl chromophores within the pigment–protein complex in the absence of red forms. While in WT at least 60% of the energy at room temperature is located on the red Chls (i.e., Chl 1015 and 1025), this is not true in the mutant, which can be observed from the fluorescence emission spectrum (34). A second factor which influences the T–S spectra can be a change in the distance between Chl 1015 and carotenoid L2 (34). It can be concluded that the lutein molecule located in the L2 site absorbs at 510 nm and is responsible for the

T–S peak at 525 nm. This is confirmed by the T–S data of Lhca4 N156F. This complex exhibits a higher amplitude at 525 nm than WT. With Chl 1012 being absent in this mutant, it is clear that the capacity to transfer triplets to L1 (neighbor of Chl 1012) is decreased, and thus, most of the triplets are transferred to the carotenoid bound to site L2. It can thus be concluded that the xanthophyll responsible for the 507 nm triplet, which shows singlet absorption at \sim 490 nm, is located in the L1 site. It is worth noting that although Chl 1012 is absent there is still substantial triplet transfer to the xanthophyll in the L1 site, indicating that Chls other than Chl 1012 can transfer triplets to this carotenoid. The candidates are Chl 1011 and Chl 1013, which are located very close to the lutein end rings in LHCII.

The “510 nm Lutein”. The data presented above clearly indicate that a lutein molecule absorbing at 510 nm is associated with LHCI. This lutein was found in Lhca3 and Lhca4 and is located in site L2. This assignment is in agreement with transient absorption data for Lhca4, showing that, upon excitation at 514 nm, most of the energy is directly transferred to the red forms [e.g., to Chl 1015 and Chl 1025 which are located near the L2 site (45)]. The T–S signal originating from this lutein molecule was found at 525 nm.

A lutein molecule absorbing at 510 nm and responsible for the 525 nm triplet spectrum was also found in trimeric LHCII associated with the L2 site (10). Interestingly, this spectral form is not present in the LHCII monomer, thus suggesting that changes in the binding site occur upon trimerization. In the case of Lhca, the 510 nm lutein is present in both monomers and dimers, indicating that the binding pocket for L2 in Lhca3 and Lhca4 complexes is very similar to that of the LHCII trimer. The origin of the strong red shift of the absorption spectrum of this carotenoid is not known; it has been proposed that the presence of Mg ions in the trimer is responsible for the large shift observed for this lutein, but in this case, the red lutein is also present in recombinant proteins which have been reconstituted in the absence of Mg (15, 46). Palacios et al. (46) proposed that the shift is due to the presence of a charged residue close to the lutein molecule. The only charged residue located relatively close to L2 in the structure of LHCII is Arg 185, which is conserved in all Lhca complexes; thus, to account for the results with Lhca, one should assume that the folding of Lhca1 and Lhca2 is different with respect to that of Lhca3 and Lhca4. One other possibility is that this xanthophyll strongly interacts with a neighbor Chl. According to the structure of LHCII, this should be Chl 1015 which lies parallel to the carotenoid in site L2. Interestingly, substitution of N, the natural ligand for Chl 1015, with H, which has been proposed to change the position of Chl 1015, induces a small blue shift in the absorption spectrum of the 510 nm lutein. Moreover, the absence of this Chl [mutant N47F (36)] leads to the loss of the 510 nm absorption (unpublished results). These findings not only confirm that the lutein responsible for the 510 nm absorption is located in the L2 site but also suggest that the lutein L2–Chl 1015 interaction is at the origin of the large observed red shift.

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